# Graphene Oxide-Reinforced Alginate/Gelatin Hydrogel via Schiffbase Bond and Thiol-Michael Addition for Bone Regeneration

Peng Ding <sup>1,†</sup>, Oseweuba Valentine Okoro <sup>2,†</sup>, Yanfang Sun <sup>3,†</sup>, Ling Wang <sup>1,†</sup>, Xiaoyan Wei <sup>4</sup>, Shuang Liu <sup>5</sup>, Yaling Deng <sup>6</sup>, Lihong Fan <sup>5</sup>, Guohua Jiang <sup>7,8</sup>, Lingling Wang <sup>9,\*</sup>, Amin Shavandi <sup>2,\*</sup> and Lei Nie <sup>1, 2,\*</sup>

College of Life Sciences, Xinyang Normal University (XYNU), Xinyang 464000,
 China;

 <sup>2</sup> Université libre de Bruxelles (ULB), École polytechnique de Bruxelles, 3BIO-BioMatter, Avenue F.D. Roosevelt, 50 - CP 165/61, 1050 Brussels, Belgium;

<sup>3</sup> College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou
 310018, China;

<sup>4</sup> Department of Medicine, Division of Endocrinology, Diabetes and Metabolism, Johns Hopkins University School of Medicine, 600 Fifth Street S., 4th floor, Rm. 4402, St. Petersburg, FL 33701, USA;

<sup>5</sup> School of Resources and Environmental Engineering, Wuhan University of Technology, Wuhan 430070, China;

<sup>6</sup> College of Intelligent Science and Control Engineering, Jinling Institute of Technology, Nanjing 211169, P.R. China;

 <sup>7</sup> School of Materials Science and Engineering, Zhejiang Sci-Tech University, Hangzhou, 310018, China;

<sup>8</sup> International Scientific and Technological Cooperation Base of Intelligent

Biomaterials and Functional Fibers, Zhejiang Sci-Tech University, Hangzhou, 310018, China;

<sup>9</sup> Analysis & Testing Center, Xinyang Normal University, Xinyang 464000, China;
 <sup>†</sup> There authors contributed equally to this work and should be considered co-first authors.

# \* Corresponding author I: Prof. Lei Nie

Post address: College of Life Sciences, Xinyang Normal University (XYNU), Xinyang 464000, China. Tel: +86-13600621068.

ORCID: 0000-0002-6175-5883

E-mail address: nieleifu@yahoo.com; nielei@xynu.edu.cn

# \* Corresponding author II: Prof. Lingling Wang

Post address: Analysis & Testing Center, Xinyang Normal University, Xinyang 464000,

China

E-mail address: wfulful@163.com

# \* Corresponding author III: Prof. Amin Shavandi

Post address: BioMatter unit - École polytechnique de Bruxelles, Université Libre de

Bruxelles, Avenue F.D. Roosevelt, 50 - CP 165/61, 1050 Brussels, Belgium

E-mail address: amin.shavandi@ulb.be

#### Abstract

The poor mechanical properties of hydrogels limit their application as scaffolds to provide support for bone regeneration. Inspired by the superior mechanical properties of nanocomposites-reinforced hydrogels and the double network (DN) structure of hydrogels, the current study investigated the fabrication of graphene oxide (GO) reinforced DN hydrogels for bone regeneration. To this regard, aldehyde methylene sodium alginate (AMSA), amino gelatin (aminoG), and dithiothreitol modified graphene oxide (DGO) were initially synthesized, and subsequently were employed in the preparation of AMSA/aminoG (AG) and DGO/AMSA/aminoG (GSG) DN-hydrogels, while imposing different mass ratios of the reacting components. The physicochemical and biological properties of the prepared hydrogels were subsequently assessed using several tests such as Fourier-transform infrared spectroscopy, X-ray photoelectron spectroscopy, nuclear magnetic resonance, cell compatibility and in vivo experiments. The compressive strengths of the prepared AG and GSG hydrogels were conversely correlated with their porosity characteristics. This study showed that although AG and GSG hydrogels had favorable cell viability and cell proliferation characteristics, GSG hydrogels presented an improved osteogenic capacity compared to AG hydrogels. This study was, therefore, able to demonstrate the functionality of employing novel GSG hydrogels in tissue engineering via their use as scaffolds for mechanical support and cell proliferation to promote bone regeneration.

# Keywords:

Hydrogels; alginate; gelatin; graphene oxide; bone regeneration.

#### **1** Introduction

Bone defects, which can be due to trauma, inflammation, osteonecrosis etc., are typically treated using bone tissue engineering approaches [1-3]. The development and application of bone tissue engineering usually involve the fabrication of functional scaffolds. This is because, scaffolds in bone tissue engineering provide a suitable environment for cell adhesion, migration, proliferation, and differentiation [1, 2, 4]. The hydrogel has three-dimensional (3D) polymer networks, contains and microenvironments similar to native extracellular matrix (ECM), which could be used as bone scaffolds [5]. However, when employed in scaffolds, these hydrogels may display suboptimal biomechanics, limiting their capacity to provide support for loadbearing bone tissues [6, 7]. The need to enhance the mechanical properties of hydrogels is therefore crucial with several strategies, such as the application of click chemistry, double network (DN), fiber/filler enforced networks etc., investigated extensively, in this regard, in the literature [7, 8].

Notably, click chemistry has been commonly used to modify natural polymers to obtain formulated hydrogels with multi-functional properties, including improved mechanical properties. Click chemistry is characterized by high reaction efficiency, high selectivity, and processing simplicity [9, 10]. Indeed, the literature reports that several click chemistries have been employed to design new hydrogels with improved properties, such as azide and alkyne cycloaddition, thiol-ene/yne addition, oxime-based click reactions, and other click reactions [9, 11]. For instance, Pupkaite *et al.*, [12] modified collagen using thiol groups, and prepared injectable collagen hydrogels by cross-linking with 8-arm polyethylene glycol-maleimide using the thiol-Michael addition click reaction. The obtained hydrogels displayed excellent shear-thinning and self-healing properties, and were cell-compatible and suitable for cell encapsulation and delivery. Similarly, Liu *et al.*, [13] fabricated *in situ*-forming dextran-based hydrogel using dextran-linked-dithiothreitol (Dex-1-DTT), glycidyl methacrylate derivatized dextran (Dex-GMA), and dithiothreitol (DTT) by thiol-Michael addition click reaction. In their study, the gelation, swelling degree and mechanical properties of the produced hydrogels were favorable and could be easily regulated by adjusting the pH of phosphate buffer saline (PBS).

In spite of these advantages, the potential of toxicity from associated catalysts, initiators and radicals, during click chemistry reactions remains concerning [14]. In this regard, it was proposed that the fabrication of a double network (DN) hydrogel could further enhance its mechanical properties while reducing toxicity issues associated with click chemistry. A DN-hydrogel is based on two interpenetrating polymer networks with contrasting physical properties [15, 16]. Usually, a weak and brittle structure is incorporated into a soft and flexible structure to form DN-hydrogel, with its toughness mainly based on the sacrificial bond principle [15, 16]. Due to the excellent load-bearing and low-friction properties, DN-hydrogels have been applied for artificial soft replacement of cartilages in several studies [17, 18]. Additionally, nanoparticles of clays, carbon nanotubes, and graphene, could be introduced into the polymer networks of hydrogels further to reinforce the hydrogels [19-24]. The resulting nanocomposite hydrogels represent a combination of polymers and nanomaterials that display

improved mechanical features [25]. Huang et al., [24] prepared pH- and temperatureresponsive DN-hydrogels using poly (N-isopropylacrylamide) (PNIPAM), and proved that the addition of clay and graphene oxide (GO) significantly improved the properties of hydrogels. Tarashi al., mechanical et [22] designed кcarrageenan/polyacrylamide (κ-Car/PAm) DN-hydrogel reinforced by GO nanosheets, the obtained hydrogel exhibited excellent mechanical strength due to the incorporation of GO, which had an enhancing contribution on the energy dissipation of DN-hydrogel. The incorporated nanoparticles could interact with polymer chains via physical adsorption, hydrogen bonding, electrostatic attraction, covalent bonding, or host-guest recognition, thus, the strength and toughness of DN-hydrogels could be further improved [26].

Recognizing the above benefits, the present study seeks to fabricate the reinforced hydrogel that, using multiple design strategies, combines the organic-inorganic double network and nanoparticles reinforce to meet the complex requirements of bone tissue. In this study, the modification of sodium alginate (SA) using 2-aminoethyl methacrylate hydrochloride (AMEM), to obtain aldehyde methylene sodium alginate (AMSA), was investigated. The grafting of gelatin using ethylenediamine (EDA) to synthesize amino gelatin (aminoG) and GO functionalization using dithiothreitol as a precursor to prepare dithiothreitol modified graphene oxide (DGO) nanosheets, were assessed. After then, the DGO nanosheets reinforced hydrogels with organic-inorganic DN structure will be constructed based on the thiol-Michael addition click reaction between DGO nanosheets and AMSA polymer, and Schiff base reactions between AMSA and aminoG

polymers (**Scheme 1**). On the one hand, the mechanical properties of hydrogels could be improved. On the other hand, the physicochemical properties of the fabricated hydrogels could be regulated, including Fourier infrared spectroscopy (FT-IR), rheological behavior, equilibrium swelling degree (ESD), microstructure, compressive strength, and degradation rate. The biological properties of the fabricated reinforced hydrogels were assessed by considering their cytocompatibility, histocompatibility and osteogenic capacity properties.



**Scheme 1**. Schematic representation of double network (DN) hydrogel using aldehyde methylene sodium alginate (AMSA), amino gelatin (aminoG), and DTT modified graphene oxide (DGO) nanosheets via thiol-Michael addition click chemistry and Schiff base reaction. (a) Synthesis of AMSA polymer. (b) Synthesis of aminoG polymer. (c) Synthesis of DGO nanosheets. (d) Formation of DGO/AMSA/aminoG (GSG) DN-hydrogels.

#### 2 Materials and methods

#### 2.1 Chemicals

Graphene oxide (GO) was purchased from Nanjing Xianfeng Nano-Materials Co., Sodium Ltd (Nanjing, China). alginate (SA,  $C_5H_7O_4COONa$ ) and Nhydroxysuccinimide (NHS) were obtained from Macklin Chemical Reagent Co., Ltd. Sodium metaperiodate (NaIO<sub>4</sub>), ethylenediamine (EDA), and 1-ethy-3-(3dimethylaminopropyl carbodiimide) hydrochloride (EDC·HCl, EDC) were purchased from Aladdin Co., Ltd (Shanghai, China). Irgacure 2959 was obtained from BASF Co., Ltd. Gelatin type A (SKU: 924504-IEA, powder) was purchased from Merck Co., Ltd. DL-Dithiothreitol (DTT) and 2-aminoethyl methacrylate hydrochloride (AMEM) were purchased from Rhawn Chemicals Reagent Co., Ltd. All other chemicals were bought from Sinopharm Chemical Reagent Co., Ltd. All chemicals and solvents purchased were used as received.

# 2.2 Polymer modifications

#### 2.2.1 Synthesis of aldehyde sodium alginate (ASA)

The aldehyde sodium alginate (ASA) was synthesized according to methods in the literature [27, 28]. Briefly, 5 g of SA was added in 500 mL of deionized water, and stirred at room temperature (RT) until complete dissolution. Then, 5.4 g of NaIO<sub>4</sub> was slowly added to SA solution in the dark at RT, and stirred (600 r/min) for 24 h. The mixture was then dialyzed using a dialysis bag (Mw 14 000) against Millipore water for 3 days. After then, the product was freeze-dried to obtain ASA. The obtained ASA was stored in the freezer (-18 °C).

#### 2.2.2 Synthesis of aldehyde methylene sodium alginate (AMSA)

AMSA was obtained using EDC/NHS condensation reaction between ASA and AMEM [28]. First, 2 g of obtained ASA was added to 200 mL of 2-(N-morpholino) ethanesulfonic acid (MES, 0.1 M) solution, and stirred for 1 h at RT. Then, 0.5 g of NHS and 1.75 g of EDC were added and stirred for 45 min under a nitrogen atmosphere. After then, 0.76 g of AMEM was added in the dark, and stirred (600 r/min) for 24 h. Excess acetone was then used to precipitate the product, and the obtained product was vacuum dried for 12 h. The dried product was then dissolved in deionized water and dialyzed (Mw 3500) against deionized water for 3 days, freeze-dried, and stored in the freezer (-18 °C).

# 2.2.3 Synthesis of amino gelatin (aminoG)

5 g of gelatin was added to 200 mL of MES solution (0.1 M), and stirred at 60 °C until forming a homogeneous solution completely. The temperature was decreased to 40 °C, and then 2 mL of EDA was added, with the resulting solution stirred for 15 min. 4.31 g of EDC and 2.59 g of NHS were subsequently added to the above solution and stirred at 40 °C for 24 h. The product was then dialyzed (Mw 3500) against deionized water for 3 days, and freeze-dried, with the dried product stored in the freezer (-18 °C).

# 2.3 Graphene oxide (GO) modifications

The DTT-modified graphene oxide (DGO) was synthesized in accordance to methods in previous works [21, 29, 30]. Briefly, GO nanosheets (0.1 g) in distilled water (100 mL) were ultrasound-treated for 3 h to obtain a stock solution (1 mg/mL). Then, 4 mL of stock solution and DTT (2.58 mM/L) were added in a glass tube heated to

90 °C under continuous magnetic stirring (600 r/min), for 30 min to obtain the DGO solution.

# 2.4 Hydrogels Fabrications

#### 2.4.1 Fabrication of AMSA/aminoG (AG) hydrogels

The AMSA/<sub>amino</sub>G (AG) hydrogels were fabricated via the Schiff base reaction between the aldehyde group in ASA and the amino group in <sub>amino</sub>G. Initially, 30 w/v% of AMSA solution and 20 w/v% <sub>amino</sub>G polymer solution were prepared. The AMSA solution and <sub>amino</sub>G solution were mixed and delivered in a glass tube after homogenizing. The mixed solution was kept for 30 min at RT to obtain AG hydrogels. The obtained AG hydrogels were designated as AG-1, AG-2, AG-3, and AG-4, depending on the mass ratios of AMSA/<sub>amino</sub>G of 1/2, 2/3, 1/1, and 3/2, respectively.

# 2.4.2 Fabrication of DGO/AMSA/aminoG (GSG) DN-hydrogels

Graphene oxide (GO) reinforced GSG DN-hydrogels were fabricated using Schiff base reaction and thiol-Michael addition click chemistry [25]. The AMSA polymer solution (30 w/v%), aminoG polymer solution (20 w/v%), DGO solution (0.5 mg/mL), and Irgacure 2959 (photoinitiator) solution were mixed and homogenized (the mass ratio of AMSA/aminoG as 3/2 was used based on the comprehensive performance of AG hydrogels using the same mass ratio) to prepare the precursor solution on the culture plate, then the solution was exposed under UV-light irradiation (45 mW/cm<sup>2</sup>) for 15 min to obtain GSG hydrogel (GSG-1). In contrast, 1 mg/mL of DGO solution was used to fabricate the GSG hydrogel, and designated as GSG-2.

### 2.5 Characterization

### 2.5.1 Fourier-transform infrared spectroscopy (FT-IR) analysis

Fourier-transform infrared spectroscopy (FT-IR, ThermoFisher, Nicolelis5) was used to analyze the presence of specific chemical groups in GO, DGO, ASA, AMSA, and <sub>amino</sub>G polymers, and the chemical interaction in AG and GSG hydrogels were investigated. The mean FT-IR spectra generated via the attenuated total reflectance (ATR) technique were recorded after an average of 64 scans and in the range of 4000 and 500 cm<sup>-1</sup>, with a resolution of 4 cm<sup>-1</sup> imposed.

## 2.5.2 X-ray Photoelectron Spectroscopy (XPS) analysis

The elemental composition of DGO nanosheets was investigated using an X-ray photoelectron spectroscopy analyzer (XPS, K-Alpha 0.05 eV, Thermo Scientific). The sample preparation procedures before XPS testing were based on our previous publication [21].

#### 2.5.3 Nuclear magnetic resonance analysis

The proton nuclear magnetic resonance (<sup>1</sup>H NMR 600 MHz NMR spectrometer, JEOL ECZ600R/S3) was used to confirm the synthesis of ASA and ASMA polymers. ASA and ASMA polymers were dissolved in deuterium oxide (D<sub>2</sub>O), and the NMR spectra were generated using a <sup>1</sup>H NMR spectrometer, equipped with a 14.09 T superconducting magnet and a 5.0 mm 600 MHz broadband Z-gradient high-resolution ROYAL probe.

#### 2.5.4 Transmission electron microscopy (TEM) analysis

Transmission electron microscopy (TEM, Tecnai G2 F20) was used to investigate

the morphology of GO and DGO nanosheets. GO, and DGO nanosheets were dispersed in distilled water, respectively, and treated ultrasonically for 30 min. Then, a drop of the suspension was dropped onto a carbon-coated copper mesh and dried under an infrared drying lamp, and the obtained samples were used for the subsequent TEM testing.

# 2.5.5 Rheology analysis

The rheological behavior of AG and GSG hydrogels was investigated using a rheometer (TA, DHR, USA). The storage modulus (G') and the loss modulus (G") of hydrogels were monitored as a function of time (strain of 1% and frequency of 1 Hz). The parallel plate geometry was 40 mm in diameter, and the hydrogel of about 2.0 mL was added onto the plate with a constant temperature of 37 °C, the time measurement was initiated within 400 s.

# 2.5.6 Equilibrium swelling analysis

The equilibrium swelling rates of the prepared hydrogels were measured using a gravimetric method at RT. Briefly, the dried as-prepared cylindrical hydrogel samples (mass of  $W_1$  in g) were fully immersed in PBS buffer until they reached swelling equilibrium. The swollen samples were taken out, the excess liquid on the samples was removed using absorbent paper, and the mass was measured as  $W_2$  in g. Then, the equilibrium swelling degree (*ESD*) of the hydrogels in PBS was calculated as follows [31];

$$ESD = \frac{W_2 - W_1}{W_1} \tag{1}$$

#### 2.5.7 Scanning electron microscopy (SEM) analysis

The morphology of the freeze-dried AG, and GSG hydrogels were investigated using scanning electron microscopy. The lyophilized hydrogels were immersed in liquid nitrogen, and cut off using a sharp blade. The cross-sections of hydrogels were sprayed with platinum for 100 s. Afterwards, the morphology was observed using a cold field emission scanning electron microscopy (SEM, Hitachi, Japan, Model S-4800). For each sample, at least 5 SEM images at different magnifications were randomly obtained, and then, the pore size was calculated using ImageJ Software.

#### 2.5.8 *Porosity analysis*

The porosity of the dried AG, and GSG hydrogels were evaluated using the liquid displacement method according to the literature [2]. Briefly, the sample was fully immersed in a known volume of ethanol ( $V_1$  in mL), and then, the total new volume was measured as  $V_2$  in mL. After 30 min, the sample was removed, and the residual volume of ethanol was measured as  $V_3$ . The porosity, P, was then calculated as follows;

$$P = \frac{V_1 - V_2}{V_2 - V_3} \times 100\% \tag{2}$$

#### 2.5.9 In vitro biodegradation analysis

The *in vitro* biodegradation of the prepared AG and GSG hydrogels was investigated by immersing the hydrogels in simulated body fluid (SBF) at 37 °C, and SBF was prepared according to previous papers [32, 33]. The mass of the samples, was measured and immersed in SBF to achieve a solid/liquid ratio of 50 mg/mL in plastic bottles and kept for 6 and 12 days without refreshing the SBF at 37 °C. The plastic bottles were sealed to avoid microbial contamination. Then, the samples were recovered via filtration, rinsed using distilled water and dried, and the final mass,  $W_2$  in g, was recorded. The degradation rate, D (mass loss), was calculated as a percentage of the initial mass,  $W_1$  in g, as follows;

$$D = \frac{W_2 - W_1}{W_1} \times 100\%$$
(3)

# 2.5.10 Compressive strength analysis

The compressive strengths of the dry AG and GSG hydrogels were tested using a mechanical machine (Zwich-Roell, Germany) fitted with a 50 N load cell at RT. The cylindrical sample (8 mm diameter and 4 mm height) was compressed to 10 % of its original height to record its stress as compressive strength, and the cross-head speed was 0.007 mm/s, and the strain rate was 10 % per min [34].

# 2.5.11 In vitro cytocompatibility via cell culture experiments

The human bone mesenchymal stem cells (hBMSCs, Normal, Human, ATCC@PCS-500-012<sup>TM</sup>) were used to evaluate the cytocompatibility of the prepared hydrogels. According to ATCC instructions, hBMSCs were cultivated using DMEM complete medium (DMEM basic medium with 10 v/v% serum and 1 v/v% mixture of streptomycin and penicillin) in a T25 cell culture flask, under a humidified atmosphere composed of 95 v/v% air and 5 v/v% CO<sub>2</sub> at 37 °C. The cells were subsequently digested using trypsin (0.25 %, Sigma-Aldrich), and the culture medium was replaced when the cell density reached 80 %. The hBMSCs at passage 5 were used for the following in vitro experiments.

#### 2.5.12 In vitro cytocompatibility via Cell Counting Kit-8 (CCK-8) analysis

The *in vitro* cytocompatibility of the prepared hydrogels was quantitatively investigated using the Cell Counting Kit-8 (CCK-8, Abcam) after culturing with hBMSCs for 1, 3, and 5 days, and the optical density (O.D) value at 570 nm was measured to indicate the presence of metabolically active hBMSCs, and cells cultured without hydrogels as a control group [35]. The prepared hydrogels were soaked in 75 v/v% of alcohol for 12 h, and washed using PBS on the ultra-clean working table. The treated samples were then added to a 48-well plate (Corning), and 1 mL of hBMSCs solution ( $1 \times 10^4$  cells/mL) was subsequently added to the plate. The plate was then incubated in a 5 % CO<sub>2</sub> atmosphere at 37 °C. The cell medium was changed every two days. After incubating for 1, 3, and 5 days, each sample was treated using a 10 µL of CCK-8 kit solution, and then incubated for 2 h at 37 °C. The treated solutions were then transferred to a 96-well plate, and the O.D value at 570 nm, was recorded using a microplate reader (Tecan GENios, Tecan Austria GmbH, Salzburg, Austria).

# 2.5.13 Fluorescent microscopy analysis

The cell morphologies of hBMSCs on hydrogels after culturing for 5 days were assessed using phalloidin-FTTC and 4'6-diamidino-2-phenylindole (DAPI) staining, DAPI and phalloidin-FTTC stain the nucleus and cytoskeleton, respectively. After the hydrogels were cultured with hBMSCs for 5 days, the hydrogels were washed using PBS and sequentially fixed with 2 v/% glutaraldehyde for 1 h. The samples were then washed using PBS and treated with 0.1 % of Triton X-100, after which phalloidin-FITC and DAPI (Thermo Scientific<sup>TM</sup>) were subsequently used according to the instruction

of the supplier. Finally, the treated hydrogels were washed twice with PBS and observed using the Confocal laser scanning microscope (CLSM, Leica TCS SP5 II, Germany).

#### 2.5.14 In vivo muscle implantation experiments

The muscle implantation was used to evaluate the histocompatibility of the prepared hydrogels, according to the literature [36, 37]. To this regard, 8-week-old Sprague–Dawley (SD) rats (330–350 g, SPF) were used and treated in accordance with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). Briefly, a muscle pouch of 8 mm long in the thigh muscle of rats was created after the rats were anaesthetized using 10 % chloral hydrate (Sigma-Aldrich) via intraperitoneal injection (3.3 mL/kg). The hydrogel was then implanted, and the wound was sutured and disinfected. After 2 and 4 weeks, the rats were euthanized, and the implanted samples were then retrieved and fixed in 4 v/v% paraformaldehyde (PFA, Aladdin, Shanghai, China) in phosphate buffer. Finally, the samples were histologically processed using hematoxylin-eosin (HE) and observed using an optical microscope.

## 2.5.15 In vivo bone regeneration evaluation

For bone regeneration evaluation of the scaffolds, a cranial defect was constructed in SD rats (330–350 g, SPF) [38]. The SD rat was anaesthetized and fixed on the operating table, followed by skin preparation and sterilization. An incision was then created on the scalp skin of the head, and the outer periosteum was subsequently moved. A round full-thickness defect of 5 mm diameter was created, on the right side of the parietal bone ring, using a bone drill, and the scaffold was implanted into the defect area. The outer periosteum and scalp skin was sutured, and the rats were treated using penicillin injection to prevent the wounds from infection in the first 3 days. After 4 weeks, the rats were euthanized, after which the samples were harvested and fixed in 4 v/v% paraformaldehyde in phosphate buffer. Lastly, HE staining was used to evaluate the bone regeneration of the prepared hydrogels as bone scaffolds.

#### 2.6 Statistical analysis

All experiments were conducted in triplicate, and the data were expressed as means  $\pm$  standard deviation. The SPSS software (SPSS Inc, Chicago IL) was used for the analysis. ANOVA statistical analyses and Tukey's test were applied to investigate specific differences. Statistical significance was defined at a p-value of < 0.05, and < 0.01 for 95 %, and 99 % confidence, respectively.

## **3** Results and discussion

# 3.1 Synthesis of DGO, AMSA, and AG

The thickness of the used traditional GO flake was around 1.25 nm [21, 29]. The transparent single-layer GO nanosheets could be observed using TEM images. **Figure 1a** shows that after GO was modified using DTT, the thickness of DGO nanosheets was increased due to the modification of GO by DTT. The FT-IR spectra analysis presented in **Figure 1b** shows that, peaks were detected at 1035 and 730 cm<sup>-1</sup> in DGO spectrum, and were due to C-S bonds and S-H bonds, respectively [29]. The peaks located at 1770 and 1690 cm<sup>-1</sup> were attributed to the C=O and C=C groups, respectively [39]. The presence of these peaks indicates that the grafting of DTT molecules on GO nanosheets via C-S bonds, occurred. **Figure 1c** also confirms the formation of C-S bonds on DGO

nanosheets using XPS analysis.



**Figure 1**. (a) TEM images of GO and DGO nanosheets. (b) FT-IR spectra of GO and DGO nanosheets. (c) XPS spectra of DGO nanosheets. (d) FT-IR spectra of SA, ASA, and AMSA polymers. (e) <sup>1</sup>H NMR spectra of ASA and AMSA polymers. (f) FT-IR spectra of gelatin and <sub>amino</sub>G polymers.

Next, NaIO<sub>4</sub> was used to oxidize SA to obtain ASA (**Scheme 1**). Compared to the previous synthesis method described [27], the yield of ASA was improved from 88 % to 92 %, while the oxidization reaction time was increased from 4 h to 24 h, and the oxidization degree was ~68 %. The peak at 1145 cm<sup>-1</sup> was observed in the FT-IR spectrum of ASA, which was attributed to the vibration of hemiacetalic groups. After then, AMSA was synthesized based on the EDC/NHS condensation reaction between ASA and AMEM. For the FT-IR spectrum of AMSA, the peak detected at 1723 cm<sup>-1</sup> was attributed to the carbonyl groups due to the grafting of AMEM on ASA via the

EDC/NHS condensation reaction (**Figure 1d**) [27]. The successful synthesis of ASA and AMSA was further confirmed using <sup>1</sup>H NMR analysis as illustrated by the signal at 5.6 ppm for ASA <sup>1</sup>H NMR spectrum, indicating the presence of newly formed methyne protons. Two new signals at 5.15 ppm corresponded to a hemiacetalic proton formed by aldehyde and neighbouring hydroxyl groups, confirming the successful modification [28]. Similarly, the <sup>1</sup>H NMR spectrum of AMSA displayed the signal at 1.9 ppm for methyl protons, and signals at 6.2 ppm and 5.7 ppm for vinyl methylene protons, indicating the successful methacrylation of ASA (**Figure 1e**). Furthermore, the presence of a slightly enhanced absorbance peak at 2943 cm<sup>-1</sup> (-CH<sub>2</sub>- group in EDA) and the peak at 1388 cm<sup>-1</sup> (bending vibration of C-H in amide III band) for the EDA-modified gelatin, indicated the successful grafting of EDA to gelatin (**Figure 1f**).

# 3.2 Fabrication of GSG DN-hydrogels

GSG hydrogels were prepared using Schiff base reaction between AMSA and aminoG, and thiol-Michael addition click chemistry between DGO and AMSA, while AG hydrogels were fabricated via the Schiff base reaction between AMSA and aminoG polymers (Scheme 1). The chemical interaction between the AG and GSG hydrogels was investigated using FT-IR, as shown in Figure S1 and Figure 2a. Figure 2a shows that for the FT-IR spectra of AG and GSG hydrogels, peaks appeared at 1724 cm<sup>-1</sup> and 1630 cm<sup>-1</sup> were attributed to the disappearance of the aldehyde group from the amino group from aminoG and the stretching vibration of C=N, due to the Schiff base reaction that employs aldehyde groups. For FT-IR spectra of GSG hydrogels, the peaks at 1547 and 1251 cm<sup>-1</sup> were due to the stretching vibrations of amino and amino C-N,

respectively [40]. In addition, the peak at 730 cm<sup>-1</sup> due to S-H groups disappeared because of thiol-Michael addition click reaction. AG and GSG hydrogels based on different crosslinking mechanism would display apparent differences in physicochemical properties.



Figure 2. (a) FT-IR spectra of AG-4, GSG-1, and GSG-2 hydrogels. (b) Rheological analysis of AG-4, GSG-1, and GSG-2 hydrogels, the storage modulus (G') and loss modulus (G'') were evaluated over time. (c) Equilibrium swelling degree (ESD) of AG-4, GSG-1, and GSG-2 hydrogels. \*p < 0.05 and \*\*p < 0.01.

The rheological behaviors of AG and GSG hydrogels were assessed using oscillatory rheology experiments. **Figure 2b** shows that the storage modulus (G') and loss modulus (G'') of AG and GSG hydrogels were stable over time. It was observed that GSG hydrogels had a great higher G' than that AG hydrogels, confirming that the rigidity of the hydrogels could be largely improved by DN structure using thiol-Michael addition click chemistry and Schiff base reaction. Also, GSG-2 displayed a higher G' than that of GSG-2, indicating that a higher DGO concentration in hydrogels resulted in a higher cross-linking degree. The equilibrium swelling degrees (ESD) of AG and GSG hydrogels were also investigated, as shown in **Figure S2** and **Figure 2c**. For the

ESD of AG hydrogels, there was a slight decrease from 779.9  $\pm$  34.9 % to 701.0  $\pm$  26.7 %, with an increasing the mass ratio of AMSA/AG from 1/2 to 3/2. However, the ESD of GSG hydrogels reduces compared to the ESD of AG hydrogels. The combination of DN structure and GO reinforced mechanism could result in a higher cross-linking degree in hydrogels, which causes an improved G' and a decreased ESD.



**Figure 3**. SEM images of (a, b) AG-1, (c, d) AG-2, (e, f) AG-3, (g, h) AG-4, (i, j) GSG-1, and (k, l) GSG-2 hydrogels at different magnification. (m) Porosity and (n) compressive strength of AG-4, GSG-1, and GSG-2 hydrogels. (o) The in vitro biodegradation rate of AG-4, GSG-1, and GSG-2 hydrogels in SBF for 12 days. \*p < 0.05 and \*\*p < 0.01.

# 3.3 Physiochemical properties

The physicochemical properties of the prepared AG and GSG hydrogels, including

microstructure, porosity, compressive strength, and in vitro biodegradability, were investigated. The cross-sectional morphology of AG and GSG hydrogels in a freezedried state was observed using SEM images (Figure 3a-l), with the results displaying an interconnected porous structure with a characteristic pore size of about 10-100 µm for all samples. The porous interconnected structure could provide enough space for vascularized bone ingrowth and encouragement of rapid bone ingrowth [41]. The pore size was calculated using ImageJ software on 10 of random SEM images for each sample (Figure S3). The pore size of AG hydrogels decreased from  $57.6 \pm 8.9 \ \mu m$  to  $46.3 \pm 11.3 \ \mu\text{m}$  as the mass ratio of AMSA/<sub>amino</sub>G increased from 1/2 to 3/2. However, the pore size of GSG hydrogels was larger than that of AG hydrogels, and sample GSG-2 (72.9  $\pm$  16.6 µm) displayed a larger pore size than GSG-1 (69.6  $\pm$  13.5 µm). Next, the porosity of the prepared hydrogels was measured, as shown in Figure S4 and Figure **3m**. The porosity of AG hydrogels had a decrease from  $76.82 \pm 2.84$  to  $71.09 \pm 2.55$  as the mass ratio of AMSA/aminoG increased from 1/2 to 3/2 (Figure S4). For GSG hydrogels, the porosity decreased compared to AG hydrogels. The mechanical properties of hydrogels were observed to be influenced by porosity characteristics [35, 42]. For instance, in all hydrogels, the compressive strengths are inversely correlated with porosity (Figure S5 and Figure 3n). Compared to AG hydrogels, the compressive strength was greatly improved from  $2.05 \pm 0.09$  MPa (AG-4) to  $8.42 \pm 0.44$  MPa (GSG-1) for GSG hydrogels, and sample GSG-2 exhibited the highest value of  $13.85 \pm 0.12$ MPa. The results also show that there is a tradeoff between porosity and mechanical strength. For instance, in GSG and AG hydrogels, the porosity decreased by 5.73 % and 2.73 %, while the compressive strengths increased by 0.27 MPa and 5.44 MPa, respectively. The ultimate compressive strength of cancellous bone was in the range of 0.7-30 MPa (1.4-5.4 MPa for cellular sawbones, and 0.7-30 MPa for trabecular structure bone) [43]. Crucially, the compressive strength of the prepared GSG samples satisfied the requirements of a porous scaffold for bone tissue engineering applications [44]. The *in vitro* biodegradability of hydrogels was investigated by soaking samples in SBF for 12 days (37 °C), and the mass loss was calculated, as shown in Figure S6 and Figure 30. During the biodegradation process, the hydrogel usually undergoes the hydrolysis phase and the crosslink junction degradation. The biodegradation rate of hydrogels increased over days, and the mass loss of AG hydrogels in SBF could reach  $54.92 \pm 3.09 \sim 58.23 \pm 5.09$  % on day 12. However, GSG hydrogels had a slower degradation rate than AG hydrogels, and sample GSG-1 displayed the lowest degradation rate (17.59  $\pm$  2.05 % on day 12) than other samples. Compared to AG hydrogels, the slower biodegradation rate of GSG hydrogels could be attributed to the DN structure of hydrogels with improved crosslink density. The results confirmed that the DN structure GSG hydrogels were relatively stable when incubated in SBF.

#### 3.4 In vitro cytocompatibility evaluation

The cytocompatibility of the AG and GSG hydrogels was evaluated using CCK-8 analysis after culturing hBMSCs with hydrogels for different days, and the results were shown in **Figure S7** and **Figure 4a**. For AG hydrogels, the viability of hBMSCs cultured on the hydrogels increased over days, however, O.D values were not greatly

influenced by the mass ratio of AMSA/aminoG. For GSG hydrogels, the number of cells proliferated on hydrogels was decreased in comparison with proliferating on AG hydrogels during the days. In addition, the cell viability on all prepared hydrogels was higher than that of a control group, confirming that the 3D micro-environment supported by the prepared hydrogels could encourage the proliferation of hBMSCs. The obtained CCK-8 results indicated the excellent cytocompatibility of AG and GSG hydrogels. On the other hand, hBMSCs cultured on AG and GSG hydrogels on day 3 was also evaluated by fluorescent microscopy images using phalloidin-FITC/DAPI staining and optical microscopy images (Figure 4b-g and Figure S8). Due to the presence of polysaccharides in both AG and GSG hydrogels, the framework of hydrogels was stained as well while staining cells. The porous interconnected structure could be apparently observed for all samples after culturing with hBMSCs for 3 days. The growth of hBMSCs in hydrogels could also be observed using optical microscopy images. Due to 3D micro-environment provided by the scaffold, most of hBMSCs were grown and diffused into the pore of the scaffold, thus, not many cells were observed using fluorescent microscopy images. Cell growth and proliferation are greatly influenced by the microstructure, and the interconnected microstructure could provide more sites and spaces for cell attachment, spread, and proliferation [45]. CCK-8 analysis and fluorescent microscopy images confirmed the excellent cytocompatibility of AG and GSG hydrogels.



Figure 4. (a) The cytocompatibility of AG-4, GSG-1, and GSG-2 hydrogels was analyzed via CCK-8 after culturing with hBMSCs for different days, without adding hydrogels as a control group. Fluorescent images (DAPI/Phalloidin-FITC) of (b) AG-1, (c) AG-2, (d) AG-3, (e) AG-4, (f) GSG-1, and (g) GSG-2 hydrogels after culturing with hBMSCs at day 3. \*p < 0.05 and \*\*p < 0.01.

#### 3.5 In vivo evaluation

The histocompatibility of the prepared hydrogels was evaluated by implanting the hydrogels into the thigh muscles of SD rats, and H&E staining images of the tissue section in the implanted site were subsequently shown in **Figure 5a-c**. After the implantation, inflammation issues, typically manifested as red swelling or effusion in the implant site, were not observed. Indeed, inflammatory cells such as neutrophils,

eosinophils, and macrophages were not detected after 2 weeks of implantation (**Figure S9**). The framework of AG-4 was incomplete due to its weak mechanical strength. Notably, some new blood vessels were observed around the GSG hydrogels, highlighting their support for vascularization. The vascularization also enhances an interconnected structure which promotes mechanical strength support. After 4 weeks of implantation, more blood vessels were observed, and interfaces between hydrogels and surrounding tissue disappeared, with more muscle tissue 'growing into' the hydrogels, especially for GSG hydrogels.

Lastly, the osteoinductivity and osteogenic capacity of hydrogels were investigated by implanting them into the cranial defect of SD rats, H&E staining images of implanted samples for 4 weeks are shown in **Figure 5d-i**. For sample AG-4, it was difficult to observe the porous structure, due to the growth of surrounding muscle tissue. For GSG hydrogels, a small amount of woven bone was apparently observed, and many osteoblasts were also found, indicating that new bone was formed. Bone regeneration usually involves osteogenesis and angiogenesis, a porous microstructure and suitable mechanical strength promoting the development of vascularization [46]. According to the cranial defect experiment, GSG hydrogels exhibited a better osteogenic capacity than AG hydrogels.



**Figure 5**. Optical micrographs of H&E staining slices of AG-4, GSG-1, and GSG-2 hydrogels after implanting in the thigh of muscle (a-c) for 4 weeks, and implanting into the cranial defect (d-i) for 4 weeks, 8-week-old SD rats were used for all experiments. Red arrows indicate new blood capillaries, blue arrows indicate muscle tissue, and purple arrows indicate osteoblasts.

#### 4 Conclusion

The present study successfully fabricated high-performance nanocomposites and DN-hydrogels that were characterized by favorable compressive strengths, *in vitro* biodegradability and biological properties. The porosity characteristics of the hydrogels,

under physiological conditions, were shown to promote cell proliferation while simultaneously providing mechanical support via scaffolds, thus highlighting their potential to promote bone regeneration. These unique properties suggest that novel designed hydrogel constitutes promising candidates as bone regeneration engineering scaffolding biomaterials. Notably, in spite of the favorable properties of the fabricated hydrogels, the present study acknowledges possible limitations due to hydrophilic expansion issues. The swelling may affect the porosity and mechanical properties and thus inhibit functionality as sufficient support for bone regeneration. Further work is therefore required in this regard.

# **Author Contributions**

The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript.

# **Conflicts of Competing Interest**

The authors declare no competing financial interest.

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